

Inhibition of HDAC3 promotes ligand-independent PPAR γ activation by protein acetylation

Xiaoting Jiang¹, Xin Ye¹, Wei Guo^{1,2}, Hongyun Lu^{1,3} and Zhanguo Gao^{1,4}

¹Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, Louisiana 70808, USA

²Department of Pathology, Shanghai University of Traditional Chinese Medicine, Shanghai, China

³Department of Endocrinology and Metabolism, The Third/Fifth Affiliated Hospital of Sun Yat-sen University, Zhuhai, Guangdong, China

⁴Department of Medical Tests, Xinxiang Medical University, Xinxiang, China

Correspondence should be addressed to Z Gao
Email
gaoz@pbrc.edu

Abstract

Peroxisome proliferator-activated receptor gamma (PPAR γ) is a nuclear receptor whose activation is dependent on a ligand. PPAR γ activation by exogenous ligands, such as thiazolidinediones (TZDs), is a strategy in the treatment of type 2 diabetes mellitus for the improvement of insulin sensitivity. In addition to a ligand, PPAR γ function is also regulated by posttranslational modifications, such as phosphorylation, sumoylation, and ubiquitination. Herein, we report that the PPAR γ protein is modified by acetylation, which induces the PPAR γ function in the absence of an external ligand. We observed that histone deacetylase 3 (HDAC3) interacted with PPAR γ to deacetylate the protein. In immunoprecipitation assays, the HDAC3 protein was associated with the PPAR γ protein. Inhibition of HDAC3 using RNAi-mediated knockdown or HDAC3 inhibitor increased acetylation of the PPAR γ protein. Furthermore, inhibition of HDAC3 enhanced the expression of PPAR γ target genes such as adiponectin and aP2. The expression was associated with an increase in glucose uptake and insulin signaling in adipocytes. HDAC3 inhibition enhanced lipid accumulation during differentiation of adipocytes. PPAR γ acetylation was also induced by pioglitazone and acetylation was required for PPAR γ activation. In the absence of TZDs, the acetylation from HDAC3 inhibition was sufficient to induce the transcriptional activity of PPAR γ . Treating diet-induced obesity mice with HDAC3 inhibitor or pioglitazone for 2 weeks significantly improved high-fat-diet-induced insulin resistance. Our results indicate that acetylation of PPAR γ is a ligand-independent mechanism of PPAR γ activation. HDAC3 inhibitor is a potential PPAR γ activator for the improvement of insulin sensitivity.

Key Words

- ▶ type 2 diabetes
- ▶ insulin sensitivity
- ▶ metabolic syndrome
- ▶ adipocytes
- ▶ adipogenesis
- ▶ PPAR γ
- ▶ posttranslational modifications
- ▶ histone deacetylase
- ▶ HDAC inhibitors
- ▶ acetylation

Journal of Molecular Endocrinology
(2014) 53, 191–200

Introduction

Peroxisome proliferator-activated receptor gamma (PPAR γ) is a well-documented transcription factor that plays an important role in the control of glucose and fatty acid metabolism. In this mechanism, PPAR γ induces the expression of adipocyte-specific genes and promotes differentiation of preadipocytes through transcriptional

activation of target genes (Rosen & Spiegelman 2000). PPAR γ is also required for the maintenance of physiological function of mature adipocytes. Insufficient PPAR γ activity is associated with adipose tissue dysfunction and glucose disorders in metabolic syndrome (Fujiki *et al.* 2009). At the molecular level, PPAR γ forms heterodimers

with the retinoid X receptor (RXR) when it binds to the promoter DNA of target genes. The transcriptional activity of PPAR γ is regulated by ligands that determine PPAR γ interaction with coactivators and corepressors (Berger & Moller 2002). Thiazolidinedione (TZD) is a synthetic PPAR γ ligand that has been widely used in clinical practice to improve insulin sensitivity in type 2 diabetes mellitus (T2DM). In the absence of ligands, PPAR γ binds the corepressor that is formed by histone deacetylase 3 (HDAC3) and silencing mediator for retinoid and thyroid hormone receptors (SMRT)/nuclear receptor corepressor (NCoR). Ligand binding leads to disassociation of the corepressor complex and induces recruitment of coactivators. Although TZDs are outstanding PPAR γ ligands with strong therapeutic activities in the treatment of T2DM, their side effects on the heart and bladder have caused alarm with regard to clinical applications. It is an urgent priority to identify a new PPAR γ activator to replace TZDs in the treatment of T2DM (Ye 2011). For this reason, we explored a new strategy of PPAR γ activation with a focus on HDAC3 inhibition.

Regulation of PPAR γ protein by direct acetylation is a new topic in the study of PPAR γ function. PPAR γ function is regulated by posttranslational modifications such as phosphorylation (Hu *et al.* 1996), sumoylation (Pascual *et al.* 2005), ubiquitination (Hauser *et al.* 2000, Floyd & Stephens 2002, Christianson *et al.* 2008, Anbalagan *et al.* 2012), and histone acetylation (Sugii & Evans 2011, Qiang *et al.* 2012). Phosphorylation of PPAR γ at serine 112 and 273 inhibits PPAR γ transcriptional activity. Sumoylation of PPAR γ at lysine 107 in the AF1 region and at lysine 395 in the AF2 region (lysine 77 and 365 in PPAR γ 1 respectively) activates PPAR γ by blocking the interaction between the NCoR of HDAC3 and PPAR γ . Ubiquitination of PPAR γ leads to protein degradation following PPAR γ activation by TZDs (Hauser *et al.* 2000, Floyd & Stephens 2002, Christianson *et al.* 2008, Anbalagan *et al.* 2012). It is largely unknown whether PPAR γ protein is acetylated and, if so, how PPAR γ function is regulated by acetylation. In this study, we addressed this issue by analysis of PPAR γ protein acetylation.

HDAC3 belongs to the class I HDAC proteins, which play important roles in the regulation of histone protein acetylation in the process of chromatin remodeling and gene transcription. HDACs have three classes, class I (HDAC1, 2, 3, 8, and 11), class II (HDAC4, 5, 6, 7, 9, and 10) (Huang *et al.* 2000), and class III (SIRT1–7) (Blander & Guarente 2004). Trichostatin-A (TSA) is a pan-HDAC inhibitor for class I and class II HDACs. In our previous studies, we reported that HDAC inhibitors such as sodium

butyrate and TSA promoted ligand-induced PPAR γ function in adipocytes *in vitro* (Gao *et al.* 2006) and prevented high-fat-diet-induced obesity (DIO) in mice (Gao *et al.* 2009). HDAC3, a member of the class I HDACs, has been reported by our and other laboratories to regulate PPAR γ function in adipocytes (Fajas *et al.* 2002, Guan *et al.* 2005, Miard & Fajas 2005, Gao *et al.* 2006). However, it is not known whether HDAC3 inhibition is sufficient to activate PPAR γ in the absence of classical ligands.

In this study, we found that PPAR γ protein is acetylated. The acetylation was induced by a ligand and decreased by HDAC3. HDAC3 inhibition induced PPAR γ acetylation and activation in the absence of exogenous ligands. The results of this study indicate that HDAC3 inhibition could be a new approach to activate PPAR γ in the absence of exogenous ligands.

Materials and methods

Mouse models and treatment

DIO male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at 16-weeks-old, and had been fed a high-fat diet (HFD, 60% calories as fat, Research Diets (New Brunswick, NJ, USA) D12492) for 10 weeks. The mice were group-housed (two to four mice per cage) in the animal facility of the Pennington Biomedical Research Center with a 12 h light:12 h darkness cycle and at a temperature of 22–24 °C. The mice had free access to water and feed. The mice were treated with HDAC3 inhibitor by i.p. injection at dose of 10 μ g/kg body weight per day for 2 weeks. Pioglitazone at a dose of 10 mg/kg body weight per day was used as a positive control. The pioglitazone was administered into the diet, and this group of mice received the same amount of DMSO in PBS by i.p. injection every day. All animal experiments were approved by the Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center.

Cell culture and reagents

The cell lines 3T3-L1 (CL-173) and HEK293 (CRL-1573) were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in 10 and 5% fetal bovine serum, DMEM in a 5% CO₂ incubator. The cells were starved in DMEM containing 0.25% fatty-acid-free BSA overnight before treatment with 150 nM of HDAC3 inhibitor. HDAC3 inhibitor (cat. no. EB1003) was purchased from KeraFAST (Boston, MA, USA). Pioglitazone (cat. no. E6910) was purchased from Sigma.

Adipogenesis

3T3-L1 preadipocytes were grown to confluence in a six-well or 100-mm plate. Then they were differentiated into adipocytes using a standard protocol. The 3T3-L1 cells were incubated in the adipogenic cocktail (5 μ g/ml insulin, 0.5 mM isobutylmethylxanthine, and 10 μ M dexamethasone) for 2 days. This was followed by incubation in an insulin-supplemented medium for an additional 4 days. The normal medium was used at day 7 to maintain the adipocytes. Adipogenesis was quantified with Oil Red O staining, as described previously (Gao *et al.* 2006).

Glucose uptake

3T3-L1 preadipocytes (5×10^5 /well) were differentiated into adipocytes in a 12-well plate. After serum starvation in 0.25% BSA DMEM overnight, the cells were treated with HDAC inhibitors, and glucose uptake was measured as described elsewhere (Gao *et al.* 2004).

Immunoblot

The whole-cell lysates were prepared by sonication in lysis buffer and used in western blotting analysis, as described elsewhere (Gao *et al.* 2002). Antibodies to acetyl-lysine (ab21623), β -actin (ab6276), HDAC3 (ab2379), and GFP (ab290) were purchased from Abcam (Cambridge, MA, USA). Monoclonal PPAR γ (E-8, sc-7273x) and HA (sc-7392) antibodies were from Santa Cruz.

Immunoprecipitation and HDAC assay

Immunoprecipitation (IP) was carried out using whole-cell lysates (400 μ g of total protein), 2–4 μ g of antibody, and 40 μ l of protein G-Sepharose beads (Amersham Biosciences), as described elsewhere (Gao *et al.* 2002). The HDAC assay was conducted using a HDAC assay kit (17-320; Millipore (Billerica, MA, USA)). Briefly, PPAR γ was immunoprecipitated and then was used as a substrate in HDAC assay. Recombinant HDAC3 protein (cat. no. H00008841, ABNOVA (Walnut, CA, USA)) was added into the reactions as an enzyme in the assay.

HDAC3 inhibitor specificity test

HDAC3 inhibitor specificity was measured using a Fluor-de-Lys HDAC3/NCOR1 fluorometric drug discovery kit (BML-AK531, Enzo Life Science (Farmingdale, NY, USA)) and a Fluor-de-Lys HDAC1 fluorometric drug discovery

assay kit (BML-AK511, Enzo Life Science) as described in the manufacturer's instructions.

I.p. insulin tolerance test

Fourteen-week-old DIO mice, which had already been given a HFD (D12492) for 8 weeks, were purchased from Jackson Laboratory (stock #000664). After quarantine, the mice were divided into three groups. Each group consisted of eight mice. For 2 weeks, 10 μ g/kg body weight per day of HDAC3 inhibitor was administered by i.p. injection. The control groups were given PBS with 0.1% DMSO (solvent). Pioglitazone was applied in the diet at the dose of 10 mg/kg body weight per day. An insulin tolerance test (ITT) was conducted by i.p. injection of insulin (I9278, Sigma) at 0.75 units/kg of body weight in mice after a 4 h fast, as described elsewhere (Gao *et al.* 2009). Blood glucose was monitored in the tail vein blood using the FreeStyle blood glucose monitoring system (TheraSense, Phoenix, AZ, USA).

Transfection and luciferase assay

Transient transfection was conducted in triplicate in 12-well plates. HEK293 cells (1.5×10^5 /well) were plated for 16 h and transfected with plasmid DNA using Lipofectamine. The PPAR γ reporter system was constituted using 0.2 μ g each of PPRE (3 \times)-luciferase, PPAR γ 2, and RXR α in each well (Gao *et al.* 2006). The cells were treated with 1 μ M pioglitazone or 150 nM HDAC3 inhibitor for 16 h to activate PPAR γ 2 after transfection for 24 h. The luciferase assay was conducted using the luciferase substrate system (Promega) with a 96-well luminometer (Gao *et al.* 2006). Each experiment was repeated at least three times.

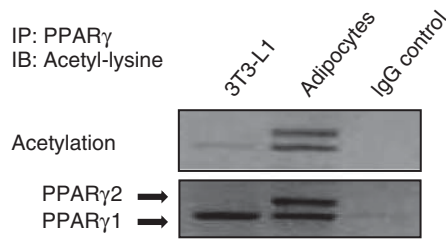
Statistical analysis

All experiments were repeated independently at least three times with consistent results. For most figures, a representative bar graph shows the mean \pm S.E.M. of multiple independent experiments normalized to appropriate controls. Student's *t*-test or one-way ANOVA was used as appropriate in statistical analyses of the data. $P < 0.05$ was considered to indicate statistical significance.

Results

PPAR γ is acetylated in adipocytes

PPAR γ function is regulated by posttranslational modifications, such as phosphorylation (Hu *et al.* 1996),

**Figure 1**

PPAR γ is an acetylated protein. PPAR γ was precipitated with a monoclonal PPAR γ antibody (E-8, sc-7273x, Santa Cruz) using 500 μ g of protein from lysates of 3T3-L1 preadipocytes and fully differentiated 3T3-L1 adipocyte. Mouse IgG was used as a negative control with the adipocyte lysates. The acetylation was detected by using rabbit acetyl-lysine antibody.

sumoylation (Yang & Gregoire 2006, Geiss-Friedlander & Melchior 2007), and ubiquitination (Hauser *et al.* 2000, Floyd & Stephens 2002, Christianson *et al.* 2008, Anbalagan *et al.* 2012). However, whether PPAR γ can be regulated by acetylation is not well known. We examined PPAR γ acetylation in 3T3-L1 adipocytes before and after differentiation. Acetylation was examined using an immunoblot with the acetylation-specific antibody after PPAR γ isolation by IP (Fig. 1). In the undifferentiated cells, the purified PPAR γ protein exhibited no significant signal in the acetylation assay (Fig. 1). In the differentiated cells, there are two isoforms of PPAR γ , PPAR γ 1 and PPAR γ 2, with different molecular weights. Both isoforms of PPAR γ proteins expressed a strong signal of acetylation (Fig. 1). The levels of acetylation were identical between PPAR γ 1 and PPAR γ 2. The results indicate that PPAR γ protein is acetylated in the adipocytes.

HDAC3 regulates PPAR γ acetylation

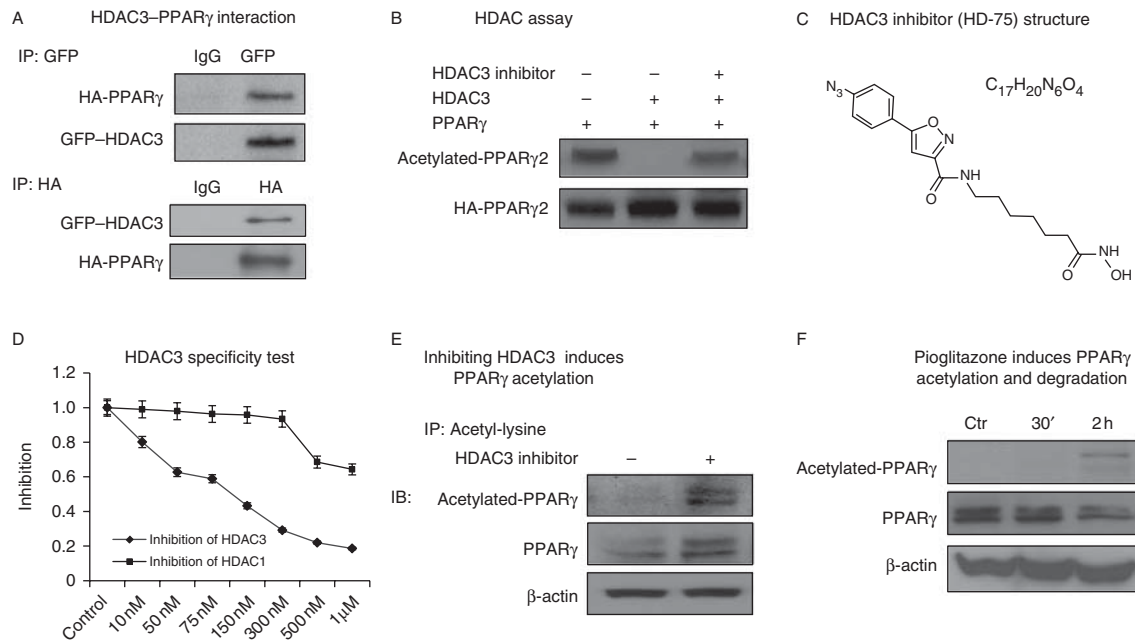
It is generally believed that PPAR γ is associated with the corepressor in the absence of a ligand. Disassociation of the corepressor complex is induced by the interaction of ligand PPAR γ , which is required for recruitment of coactivators and acetylation of histone proteins in the initiation of gene transcription. The corepressor contains HDAC3 and SMRT or NCoR. HDAC3 inhibits transcription by deacetylating histone proteins (Zamir *et al.* 1997, Treuter *et al.* 1998, Hu *et al.* 2001, Krogsdam *et al.* 2002, Gao *et al.* 2005). However, it is not known whether HDAC3 regulates acetylation of PPAR γ protein. To address this issue, we examined HDAC3–PPAR γ interaction in cells through IP. In the study, GFP-tagged HDAC3 was expressed, together with HA-tagged PPAR γ , in a transient cotransfection of HEK293 cells. Antibodies to GFP and HA

were used to isolate HDAC3 and PPAR γ in IP respectively. We observed that the HDAC3 product contained PPAR γ and that PPAR γ product contained HDAC3 (Fig. 2A). The results indicate that HDAC3 physically interacts with PPAR γ . To test HDAC3 in deacetylation of PPAR γ , we determined their enzyme and substrate relationship. In the assay, a recombinant HDAC3 protein (cat. no. H00008841, ABNOVA) was used as the deacetylase enzyme. Acetylation was induced in HA-tagged PPAR γ 2 in HEK 293 cells with the HDAC inhibitor TSA (200 nM for 30 min). When HDAC3 was inhibited with a chemical inhibitor (HD-75) in a deacetylation assay in test tube, PPAR γ acetylation was preserved by the inhibitor (Fig. 2B). The acetylation was reduced in the absence of the inhibitor (Fig. 2B). The results indicate that as a protein deacetylase, HDAC3 directly deacetylates PPAR γ protein.

We tested the specificity of the HDAC3 inhibitor HD-75 (Fig. 2C) using a Fluor-de-Lys HDAC3/NCOR1 fluorometric drug discovery kit and a Fluor-de-Lys HDAC1 fluorometric drug discovery assay kit. A HDAC1 assay kit was used as a control for the specificity of the HDAC3 inhibitor. These kits are ideal for chemical library screening of candidate inhibitors. In the HDAC3 assay, the deacetylase activity of HDAC3 was inhibited by the HDAC3 inhibitor at IC₅₀ of 150 nM. At this dosage, it only slightly inhibited HDAC1 activity (Fig. 2D). This indicates that HD-75 has specificity for HDAC3.

PPAR γ acetylation was examined using the inhibition of HDAC3 in 3T3-L1 adipocytes. We tested whether HD-75 causes a strong induction of PPAR γ acetylation at a dose of 150 nM in 3T3-L1 adipocytes. In the study, 3T3-L1 adipocytes were treated with 150 nM of HDAC3 inhibitor for 2 h. The cells were harvested and 500 μ g of the whole-cell lysates protein was used in IP with anti-acetyl-lysine antibody. We detected a stronger PPAR γ signal in the IP product in the sample treated with the HDAC3 inhibitor. PPAR γ acetylation was significantly enhanced by the HDAC3 inhibitor and also in the immunoblots (Fig. 2E). Briefly, 40 μ g of protein from 500 μ g/500 μ l IP samples was used for the loading control indicated by the β -actin in the immunoblot (Fig. 2E, bottom panel). The results indicate that PPAR γ acetylation was regulated by the HDAC3 inhibitor in cells.

PPAR γ activation by the ligands has been reported to induce degradation of the PPAR γ protein (Hauser *et al.* 2000, Floyd & Stephens 2002). It is not known whether the ligand induces PPAR γ acetylation. We addressed this question by examining PPAR γ acetylation in pioglitazone-treated 3T3-L1 adipocytes. In cells, pioglitazone enhanced PPAR γ acetylation (Fig. 2F). The results indicate that

**Figure 2**

HDAC3 regulates PPAR γ acetylation. (A) Physical interaction of PPAR γ with HDAC3. Co-IP of HA-tagged PPAR γ or GFP-tagged HDAC3 in HEK293 cells. (B) HDAC3 deacetylates PPAR γ in HDAC assay. HA-PPAR γ 2 plasmids were transfected into HEK293 cells. The cells were treated with 200 nM TSA for 30 min to induce protein acetylation. PPAR γ was immunoprecipitated on beads, and the IP product on beads served as a substrate in the HDAC assay.

(C) HDAC3 inhibitor structure. (D) HDAC3 specificity test. Ten nanograms of HDAC3 protein was used in the HDAC3 assay. (E) Inhibiting HDAC3 induces PPAR γ acetylation. 3T3-L1 adipocytes were treated with 200 nM TSA for 30 min to induce protein acetylation. PPAR γ was immunoprecipitated on beads, and the IP product on beads served as a substrate in the HDAC assay. (F) Pioglitazone induces PPAR γ acetylation and degradation in adipocytes.

pioglitazone induces PPAR γ acetylation. Acetylation of PPAR γ by the inhibition of HDAC3 did not cause PPAR γ degradation. This indicates that acetylation of PPAR γ by the inhibition of HDAC3 may be different from pioglitazone-induced acetylation of PPAR γ .

HDAC3 inhibitor induces PPAR γ acetylation at multiple lysine sites

The PPAR γ acetylation sites were analyzed by mass spectrometry. In the study, PPAR γ was expressed in a transient transfection of HEK293 cells. Antibody to PPAR γ was used to isolate PPAR γ in IP. PPAR γ in the IP product was purified by SDS-PAGE. These analyses were performed by the Proteomics Core facility at Applied Biomics, Inc. (23785 Cabot Blvd., Suite 311, Hayward, CA, USA). We used their service and detected four acetylated lysine residues in PPAR γ (Fig. 3). The four acetylated lysine sites (K289, K386, K462, and K466) were detected by mass spectrometry (lower panel). The data confirmed that PPAR γ acetylation was induced by the HDAC3 inhibitor. Further study is needed of the function of acetylated lysine sites in PPAR γ .

Acetylation induces PPAR γ function

To test acetylation modification in the regulation of PPAR γ function, we determined the transcriptional activity of PPAR γ by quantifying the expression of the target genes. PPAR γ acetylation was induced in 3T3-L1 adipocytes through the inhibition of HDAC3 activity with gene knockdown. A vector-based GFP-positive RNAi to HDAC3 was delivered by adenovirus, which infected 3T3-L1 adipocytes with 90% efficiency (Fig. 4A). The efficiency was quantified for GFP-positive cells under a fluorescence microscope. The expression of PPAR γ target genes was determined using an immunoblot. In the control cells that were infected with the control virus, HDAC3 protein was observed to correspond to an abundant protein band. In the cells infected by RNAi virus, HDAC3 was reduced by 90% according to the decreased signal of the HDAC3 protein band (Fig. 4B). Knockdown of HDAC3 significantly increased expression of PPAR γ -responsive genes, including aP2 (fatty acid-binding protein 4 (FABP4); Fig. 4B). The results indicate that HDAC3 inhibition promotes the transcriptional activity of PPAR γ in adipocytes.

HDAC3 knockdown promotes adipogenesis. PPAR γ induces the expression of a variety of genes in the pathways

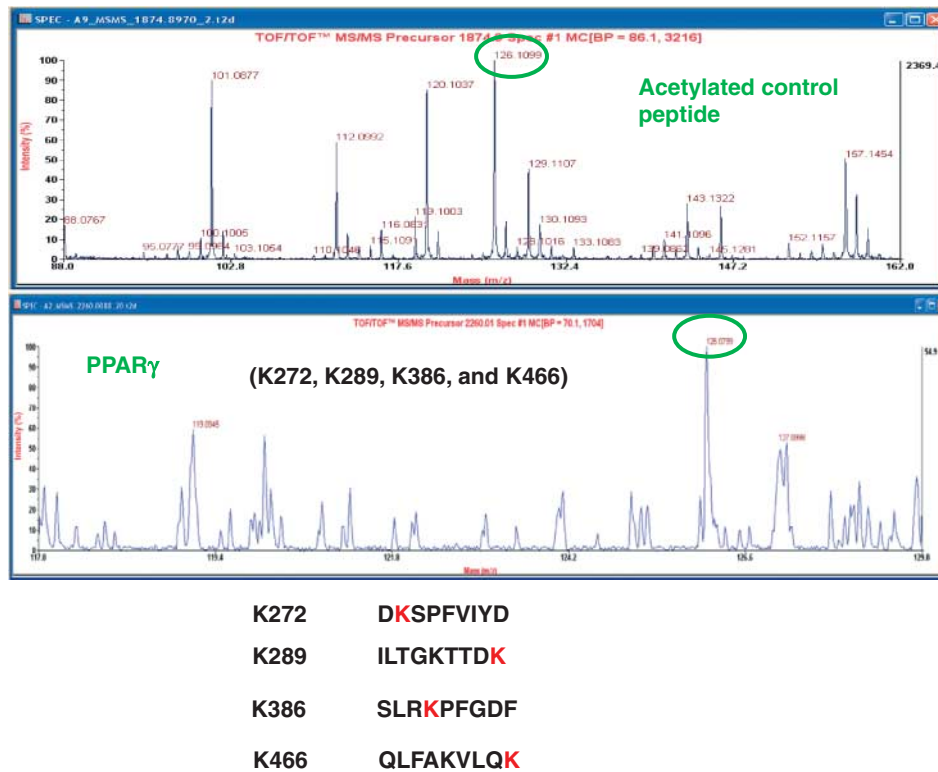


Figure 3

Acetylation sites analysis by mass spectrometry. Acetylated control peptide was generated by mass spectrometry at a 126.1 peak (top panel). Four acetylated lysine sites (K289, K386, K462, and K466) were detected

by MS (lower panel). The MS figure shows one of the four acetylated lysine sites. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0066>.

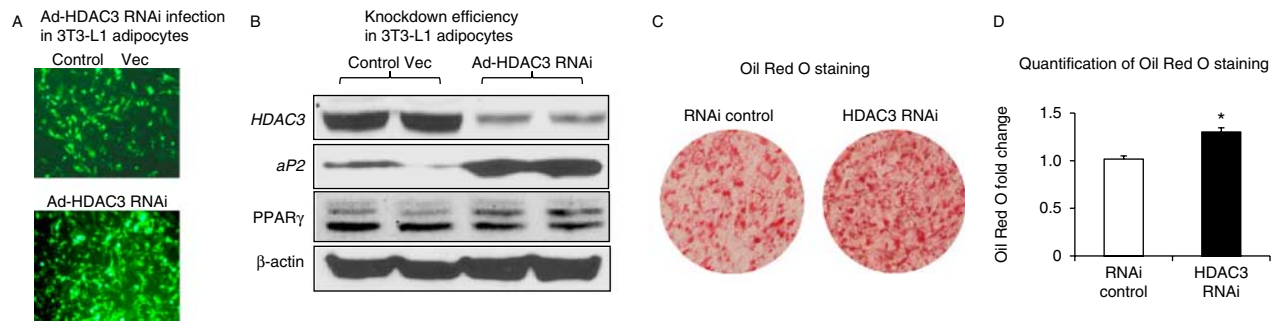
for lipid biosynthesis and storage, which is required for the differentiation of preadipocytes. Adipogenesis is often used to determine PPAR γ function. We induced adipogenesis to test the effects of HDAC3 inhibition on the regulation of PPAR γ function. HDAC3 was inhibited in 3T3-L1 preadipocytes by gene knockdown using the adenovirus RNAi delivery system (Fig. 4C).

Adipogenesis was induced at 48 h after virus infection. We observed the green fluorescence in cells from days 1 to 8 during adipogenesis (data not shown). Lipid accumulation was quantified by Oil Red O staining in the differentiated cells 8 days later. HDAC3 knockdown increased the lipid content by 30% in this adipogenesis system (Fig. 4D). The results indicate that inhibition of HDAC3 promotes lipid accumulation in adipocytes. The result further supports the hypothesis that inhibition of HDAC3 could be an approach to promote PPAR γ function.

HDAC3 inhibitor promotes adipogenesis

A chemical inhibitor of HDAC3 was tested for the regulation of adipogenesis in an effort to identify a new

PPAR γ activator independent of TZDs. In the study, activation of PPAR γ by the HDAC3 inhibitor was tested in the adipocyte differentiation model. The 3T3-L1 adipocytes were differentiated in the standard adipogenic cocktail. The HDAC3 inhibitor was added to the culture medium at a concentration of 150 nM during adipogenesis. Pioglitazone was used as a positive control. At the end of differentiation, the degree of differentiation was determined by Oil Red O staining of intracellular lipids. The HDAC3 inhibitor enhanced lipid accumulation in the cells, as indicated by the results (Fig. 5A). Oil Red O staining was enhanced by 50% in the inhibitor- and pioglitazone-treated groups (Fig. 5A, lower panel). The HDAC3 inhibitor had the same effect as pioglitazone in inducing adipogenesis. The PPAR γ target genes including adiponectin and aP2 were enhanced by the inhibitor and pioglitazone in the western blotting analysis (Fig. 5B). Comparing the HDAC3 inhibitor with pioglitazone, the HDAC3 inhibitor generated a greater increase in adiponectin expression. Adiponectin is known to promote insulin sensitivity. In a reporter assay, the HDAC3 inhibitor had the same effect as pioglitazone in inducing

**Figure 4**

Acetylation induces PPAR γ activation. (A) 3T3-L1 adipocytes were infected with adeno-GFP-HDAC3 RNAi virus. (B) Knockdown efficiency of *HDAC3* in 3T3-L1 adipocytes with a 24-h infection and expression of the PPAR γ target gene *aP2* were measured using an immunoblot. (C) Knockdown of *HDAC3* promotes adipogenesis. 3T3-L1 preadipocytes were infected with 50 μ l of adenovirus in a 10-cm cell dish for 24 h, and then the cells were induced for

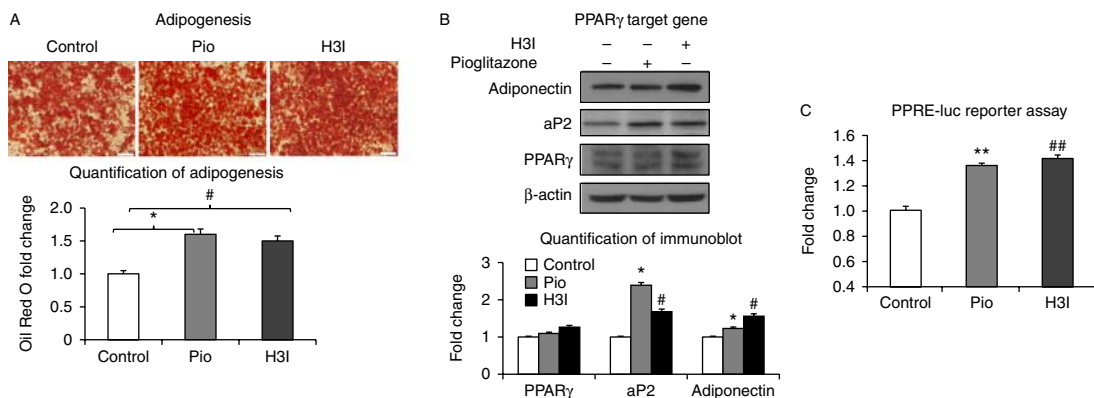
adipogenesis with a standard protocol. After 8 days of induction, the cells were stained with Oil Red O. Adeno-RNAi empty vector virus was used as a negative control. (D) Adipogenesis was quantified using a microscope and color absorbance. Data presented from triplicates analysed by Student's *t*-test. * $P < 0.05$. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0066>.

PPAR γ transcriptional activity (Fig. 5C). The results indicate that the HDAC3 inhibitor activates PPAR γ function in the absence of exogenous ligands and that the potency of the HDAC3 inhibitor is similar to that of pioglitazone.

Inhibition of HDAC3 enhances insulin sensitivity

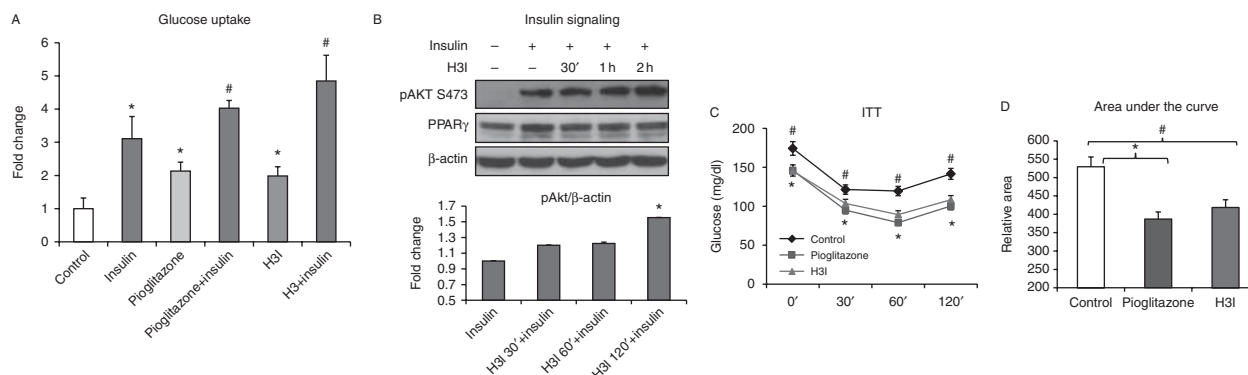
To further investigate the effect of HDAC3 inhibitor in the activation of PPAR γ , first, we used glucose uptake to determine PPAR γ function, which may enhance glucose uptake by induction of IRS2 and Glut4 in the insulin-signaling pathway. Pioglitazone was used as a positive control. The results indicated that PPAR γ ligand increased

insulin-induced glucose uptake in 3T3-L1 adipocytes (Fig. 6A). The HDAC3 inhibitor exhibited the same activity in the promotion of glucose uptake (Fig. 6A). Insulin signaling activity was examined by AKT serine 473 phosphorylation. Insulin-induced phosphorylation of AKT serine 473 was enhanced by the HDAC3 inhibitor (Fig. 6B). To test whether HDAC3 inhibitor enhances insulin sensitivity *in vivo*, we treated DIO mice with HDAC3 inhibitor at 10 a dose of μ g/kg body weight per day for 2 weeks. The treatment was given to 16-week-old DIO mice, which had been fed a HFD for 10 weeks. In our previous studies, feeding B6 mice for 12 weeks with a HFD induced insulin resistance. Pioglitazone at the dose of 10 mg/kg body weight per day was used as a positive control. The pioglitazone was

**Figure 5**

Inhibiting HDAC3-promoted PPAR γ function. (A) Adipogenesis. H3I (150 nM) was added into a standard inducing cocktail, and adipogenesis was induced in a 12-well plate. Adipogenesis was evaluated using Oil Red O staining methods. The experiments were conducted at least three times, and each trial had consistent results. (B) PPAR γ target gene expression by

western blotting and quantification. (C) PPRE-Luc reporter assay. H3I vs control, * $P < 0.05$, ** $P < 0.001$; Pio vs control, * $P < 0.05$, ** $P < 0.001$ by Student's *t*-test. Pio, pioglitazone; H3I, HDAC3 inhibitor. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0066>.

**Figure 6**

Inhibition of HDAC3 increased glucose uptake and insulin sensitivity.

(A) Glucose uptake. Fully differentiated 3T3-L1 adipocytes were treated with H3I overnight, and then glucose uptake was measured. *Drug vs control;

#drug+insulin vs insulin; $P < 0.05$ by Student's *t*-test. H3I, HDAC3 inhibitor.

(B) Insulin signaling. (C) Insulin tolerance test. (D) Area under the curve of ITT.

#H3I vs control; *pioglitazone vs control; *, # $P < 0.05$ by Student's *t*-test, $n = 8$.

administered into the diet, and this group of mice received the same amount of DMSO in PBS by i.p. injection every day. After 2 weeks of treatment, insulin sensitivity was evaluated by an ITT after 4 h of fasting. The HDAC3 inhibitor and pioglitazone both significantly reduced glucose levels and enhanced insulin sensitivity (Fig. 6C and D). Body weight and food intake were not changed in the mice by the 2-week treatment. The results indicate that the HDAC3 inhibitor enhanced PPAR γ function, serving as an insulin sensitizer in adipocytes.

Discussion

Our results indicate that acetylation of PPAR γ is induced by pioglitazone. Pioglitazone induces PPAR γ activation through recruitment of coactivators and disassociation of corepressors. The coactivators contribute to the transcriptional activation by inducing histone acetylation, which is required for chromatin structure change. There has been little information available about PPAR γ acetylation, though PPAR γ activity is regulated by protein modification such as phosphorylation (Hu *et al.* 1996), sumoylation (Pascual *et al.* 2005), and ubiquitination (Hauser *et al.* 2000, Floyd & Stephens 2002, Christianson *et al.* 2008, Anbalagan *et al.* 2012). In a recent study, it was reported that PPAR γ acetylation (lysine 268 and 293) was induced by a ligand and decreased by SIRT1 in HEK293 cells (Qiang *et al.* 2012). Although the results of that study indicated a role of acetylation in the regulation of PPAR γ function, it was not known whether the acetylation happens in the absence of a ligand. In that study, PPAR γ acetylation was regulated by SIRT1 and the effect was investigated in 'browning' of white adipose tissue. The acetylation

inhibits brown adipocyte differentiation in the white adipose tissue (inguinal fat) by blocking the interaction of PPAR γ with the coactivator PRDM16. It was reported that inhibition of the acetylation promoted preadipocyte differentiation into brown adipocytes in response to cold challenge at 4 °C in a cold room. The results indicated that PPAR γ acetylation favors lipid accumulation and preadipocyte differentiation into white adipocytes, which disfavors brown adipocyte differentiation. HDAC1/HDAC3 was reported to modulate PPAR γ transcription through the sumoylated CEBPD in hepatic lipogenesis (Lai *et al.* 2008). However, there is no report that PPAR γ was acetylated by HDAC1/HDAC3. Our results indicate that HDAC3 regulates PPAR γ acetylation and function directly. In this current study, we observed that PPAR γ acetylation was induced by pioglitazone (Fig. 2F). PPAR γ acetylation was induced by the inhibition of HDAC3 in the absence of an exogenous ligand. The ligand-independent acetylation enhanced the transcriptional activity of PPAR γ , as indicated by PPAR γ target gene expression, lipid accumulation in adipogenesis, and insulin-induced uptake. Our results indicate that PPAR γ acetylation could be a new approach to increase PPAR γ activity and that this acetylation may occur in the absence of exogenous ligands. It is not known whether acetylation correlates with phosphorylation, sumoylation, and ubiquitination of the PPAR γ protein.

In terms of the mechanism by which PPAR γ acetylation leads to PPAR γ activation, we would like to propose a model here. In this model, we suggest that there is a basal level of acetylation of the PPAR γ protein in the absence of a ligand. The corepressor removes the acetylation constantly to prevent PPAR γ activation without a ligand.

The corepressor activity is abolished when it is dissociated from the PPAR γ protein in response to a ligand. When the corepressor activity is inhibited, the acetylation accumulates in the PPAR γ protein in the absence of deacetylation activity. Acetylated PPAR γ induces the recruitment of acetyltransferases (HATs), such as p300/CBP, to induce gene transcription, which in turn induces histone acetylation in PPAR γ -responsive genes (Graves *et al.* 1992, Freedman 1999, Rosen & Spiegelman 2000, Rosen *et al.* 2000). This model explains the role of PPAR γ acetylation in PPAR γ activation in the absence of a ligand. Our results indicate that PPAR γ acetylation is coupled with histone acetylation. Histone acetylation is required for gene transcription, but histone acetylation is probably a consequence of PPAR γ acetylation. This possibility remains to be tested.

Our results indicate that the HDAC3 inhibitor is a new PPAR γ activator that exhibits potency similar to that of pioglitazone. The TZDs are the most powerful insulin sensitizer in the treatment of T2DM (Tontonoz & Spiegelman 2008). TZD-based medicines include pioglitazone (Actos by Takeda Pharmaceuticals (Chuo-ku, Osaka, Japan)) and rosiglitazone (Avandia by GlaxoSmithKline). Although TZD-based medicines have outstanding therapeutic activities, the adverse effects, such as heart attacks and bladder cancer, have significantly reduced their applications in the treatment of T2DM (Rosen 2010, Ferrara *et al.* 2011, Cariou *et al.* 2012, Tseng 2012). We believe that PPAR γ activation remains an excellent approach in the treatment of T2DM. All of the TZD-based medicines activate PPAR γ . However, their side effects are different for the heart and bladder, indicating that the side effects are not due to PPAR γ activation. It is likely that the side effects are the off-target activities of the medicine. Our results indicate that the HDAC3 inhibitor is a potential new generation of PPAR γ activator and an insulin sensitizer. Inhibition of HDAC3 is beneficial in preventing neuronal death (Bardai *et al.* 2012), improving β -cell function (Chou *et al.* 2012), and having anticancer effects (Escaffit *et al.* 2007, Bhaskara *et al.* 2008). Moreover, inhibition of HDAC3 promotes the transcriptional activities of myocyte enhancer factor 2 (Naya & Olson 1999, Gregoire *et al.* 2007), implying that inhibition of HDAC3 may protect heart function. In our previous study, pan-inhibitors of HDACs prevented HFD-induced obesity and insulin resistance in mice (Gao *et al.* 2009). These findings indicate that HDAC3 inhibitor may be able to avoid the side effects of synthetic PPAR γ ligands *in vivo*.

In conclusion, we report that the transcription factor PPAR γ is modulated by acetylation in response to ligands.

The acetylation is sufficient to induce PPAR γ function in the absence of exogenous ligands. A HDAC3 inhibitor is able to activate PPAR γ in a ligand-independent manner. These results indicate that the HDAC3 inhibitor may be a ligand-independent PPAR γ activator. Inhibition of HDAC3 may represent a new approach for improving insulin sensitivity in the treatment of T2DM.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by National Institutes of Health Centers of Biomedical Research Excellence (COBRE) grant (2P20RR021945), American Diabetes Association Junior Faculty Award 1-09-JF-17, and the National Natural Science Foundation of China (grant number 81370915).

References

- Anbalagan M, Huderson B, Murphy L & Rowan BG 2012 Post-translational modifications of nuclear receptors and human disease. *Nuclear Receptor Signaling* **10** e001. (doi:10.1621/nrs.10001)
- Bardai FH, Price V, Zayman M, Wang L & D'Mello SR 2012 Histone deacetylase (HDAC1) is a molecular switch between neuronal survival and death. *Journal of Biological Chemistry* **287** 35444–35453. (doi:10.1074/jbc.M112.394544)
- Berger J & Moller DE 2002 The mechanisms of action of PPARs. *Annual Review of Medicine* **53** 409–435. (doi:10.1146/annurev.med.53.082901.104018)
- Bhaskara S, Chyla BJ, Amann JM, Knutson SK, Cortez D, Sun ZW & Hiebert SW 2008 Deletion of *histone deacetylase 3* reveals critical roles in S phase progression and DNA damage control. *Molecular Cell* **30** 61–72. (doi:10.1016/j.molcel.2008.02.030)
- Blander G & Guarente L 2004 The SIR2 family of protein deacetylases. *Annual Review of Biochemistry* **73** 417–435. (doi:10.1146/annurev.biochem.73.011303.073651)
- Cariou B, Charbonnel B & Staels B 2012 Thiazolidinediones and PPAR γ agonists: time for a reassessment. *Trends in Endocrinology and Metabolism* **23** 205–215. (doi:10.1016/j.tem.2012.03.001)
- Chou DH, Holson EB, Wagner FF, Tang AJ, Maglathlin RL, Lewis TA, Schreiber SL & Wagner BK 2012 Inhibition of histone deacetylase 3 protects β cells from cytokine-induced apoptosis. *Chemistry & Biology* **19** 669–673. (doi:10.1016/j.chembiol.2012.05.010)
- Christianson JL, Nicoloso S, Straubhaar J & Czech MP 2008 Stearoyl-CoA desaturase 2 is required for peroxisome proliferator-activated receptor γ expression and adipogenesis in cultured 3T3-L1 cells. *Journal of Biological Chemistry* **283** 2906–2916. (doi:10.1074/jbc.M705656200)
- Escaffit F, Vaute O, Chevillard-Briet M, Segui B, Takami Y, Nakayama T & Trouche D 2007 Cleavage and cytoplasmic relocalization of histone deacetylase 3 are important for apoptosis progression. *Molecular and Cellular Biology* **27** 554–567. (doi:10.1128/MCB.00869-06)
- Fajas L, Egler V, Reiter R, Hansen J, Kristiansen K, Debril MB, Miard S & Auwerx J 2002 The retinoblastoma–histone deacetylase 3 complex inhibits PPAR γ and adipocyte differentiation. *Developmental Cell* **3** 903–910. (doi:10.1016/S1534-5807(02)00360-X)
- Ferrara A, Lewis JD, Quesenberry CP Jr, Peng T, Strom BL, Van Den Eeden SK, Ehrlich SF & Habel LA 2011 Cohort study of pioglitazone and cancer

- incidence in patients with diabetes. *Diabetes Care* **34** 923–929. (doi:10.2337/dc10-1067)
- Floyd ZE & Stephens JM 2002 Interferon- γ -mediated activation and ubiquitin–proteasome-dependent degradation of PPAR γ in adipocytes. *Journal of Biological Chemistry* **277** 4062–4068. (doi:10.1074/jbc.M108473200)
- Freedman LP 1999 Increasing the complexity of coactivation in nuclear receptor signaling. *Cell* **97** 5–8. (doi:10.1016/S0092-8674(00)80708-4)
- Fujiki K, Kano F, Shiota K & Murata M 2009 Expression of the peroxisome proliferator activated receptor γ gene is repressed by DNA methylation in visceral adipose tissue of mouse models of diabetes. *BMC Biology* **7** 38. (doi:10.1186/1741-7007-7-38)
- Gao Z, Hwang D, Bataille F, Lefevre M, York D, Quon MJ & Ye J 2002 Serine phosphorylation of insulin receptor substrate 1 by inhibitor κ B kinase complex. *Journal of Biological Chemistry* **277** 48115–48121. (doi:10.1074/jbc.M209459200)
- Gao Z, Zhang X, Zuberi A, Hwang D, Quon MJ, Lefevre M & Ye J 2004 Inhibition of insulin sensitivity by free fatty acids requires activation of multiple serine kinases in 3T3-L1 adipocytes. *Molecular Endocrinology* **18** 2024–2034. (doi:10.1210/me.2003-0383)
- Gao Z, Chiao P, Zhang X, Lazar MA, Seto E, Young HA & Ye J 2005 Coactivators and corepressors of NF- κ B in I κ B α gene promoter. *Journal of Biological Chemistry* **280** 21091–21098. (doi:10.1074/jbc.M500754200)
- Gao Z, He Q, Peng B, Chiao PJ & Ye J 2006 Regulation of nuclear translocation of HDAC3 by I κ B α is required for tumor necrosis factor inhibition of peroxisome proliferator-activated receptor γ function. *Journal of Biological Chemistry* **281** 4540–4547. (doi:10.1074/jbc.M507784200)
- Gao Z, Yin J, Zhang J, Ward RE, Martin RJ, Lefevre M, Cefalu WT & Ye J 2009 Butyrate improves insulin sensitivity and increases energy expenditure in mice. *Diabetes* **58** 1509–1517. (doi:10.2337/db08-1637)
- Geiss-Friedlander R & Melchior F 2007 Concepts in sumoylation: a decade on. *Nature Reviews Molecular Cell Biology* **8** 947–956. (doi:10.1038/nrm2293)
- Graves RA, Tontonoz P & Spiegelman BM 1992 Analysis of a tissue-specific enhancer: ARF6 regulates adipogenic gene expression. *Molecular and Cellular Biology* **12** 1202–1208. (doi:10.1128/MCB.12.3.1202)
- Gregoire S, Xiao L, Nie J, Zhang X, Xu M, Li J, Wong J, Seto E & Yang XJ 2007 Histone deacetylase 3 interacts with and deacetylates myocyte enhancer factor 2. *Molecular and Cellular Biology* **27** 1280–1295. (doi:10.1128/MCB.00882-06)
- Guan HP, Ishizuka T, Chui PC, Lehrke M & Lazar MA 2005 Corepressors selectively control the transcriptional activity of PPAR γ in adipocytes. *Genes and Development* **19** 453–461. (doi:10.1101/gad.1263305)
- Hauser S, Adelmant G, Sarraf P, Wright HM, Mueller E & Spiegelman BM 2000 Degradation of the peroxisome proliferator-activated receptor γ is linked to ligand-dependent activation. *Journal of Biological Chemistry* **275** 18527–18533. (doi:10.1074/jbc.M001297200)
- Hu E, Kim JB, Sarraf P & Spiegelman BM 1996 Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPAR γ . *Science* **274** 2100–2103. (doi:10.1126/science.274.5295.2100)
- Hu X, Li Y & Lazar MA 2001 Determinants of CoRNR-dependent repression complex assembly on nuclear hormone receptors. *Molecular and Cellular Biology* **21** 1747–1758. (doi:10.1128/MCB.21.5.1747-1758.2001)
- Huang EY, Zhang J, Miska EA, Guenther MG, Kouzarides T & Lazar MA 2000 Nuclear receptor corepressors partner with class II histone deacetylases in a Sin3-independent repression pathway. *Genes and Development* **14** 45–54. (doi:10.1621/nrs.10001)
- Krogsgaard AM, Nielsen CA, Neve S, Holst D, Helledie T, Thomsen B, Bendixen C, Mandrup S & Kristiansen K 2002 Nuclear receptor corepressor-dependent repression of peroxisome-proliferator-activated receptor δ -mediated transactivation. *Biochemical Journal* **363** 157–165. (doi:10.1042/0264-6021:3630157)
- Lai PH, Wang WL, Ko CY, Lee YC, Yang WM, Shen TW, Chang WC & Wang JM 2008 HDAC1/HDAC3 modulates PPARG2 transcription through the sumoylated CEBPD in hepatic lipogenesis. *Biochimica et Biophysica Acta* **1783** 1803–1814. (doi:10.1016/j.bbamcr.2008.06.008)
- Miard S & Fajas L 2005 Atypical transcriptional regulators and cofactors of PPAR γ . *International Journal of Obesity* **29** (Suppl 1) S10–S12. (doi:10.1038/sj.ijo.0802906)
- Naya FJ & Olson E 1999 MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation. *Current Opinion in Cell Biology* **11** 683–688. (doi:10.1016/S0955-0674(99)00036-8)
- Pascual G, Fong AL, Ogawa S, Gamliel A, Li AC, Perissi V, Rose DW, Willson TM, Rosenfeld MG & Glass CK 2005 A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR- γ . *Nature* **437** 759–763. (doi:10.1038/nature03988)
- Qiang L, Wang L, Kon N, Zhao W, Lee S, Zhang Y, Rosenbaum M, Zhao Y, Gu W, Farmer SR *et al.* 2012 Brown remodeling of white adipose tissue by SirT1-dependent deacetylation of Ppar γ . *Cell* **150** 620–632. (doi:10.1016/j.cell.2012.06.027)
- Rosen CJ 2010 Revisiting the rosiglitazone story – lessons learned. *New England Journal of Medicine* **363** 803–806. (doi:10.1056/NEJMp1008233)
- Rosen ED & Spiegelman BM 2000 Molecular regulation of adipogenesis. *Annual Review of Cell and Developmental Biology* **16** 145–171. (doi:10.1146/annurev.cellbio.16.1.145)
- Rosen ED, Walkey CJ, Puigserver P & Spiegelman BM 2000 Transcriptional regulation of adipogenesis. *Genes and Development* **14** 1293–1307. (doi:10.1101/gad.14.11.1293)
- Sugii S & Evans RM 2011 Epigenetic codes of PPAR γ in metabolic disease. *FEBS Letters* **585** 2121–2128. (doi:10.1016/j.febslet.2011.05.007)
- Tontonoz P & Spiegelman BM 2008 Fat and beyond: the diverse biology of PPAR γ . *Annual Review of Biochemistry* **77** 289–312. (doi:10.1146/annurev.biochem.77.061307.091829)
- Treuter E, Albrechtsen T, Johansson L, Leers J & Gustafsson JA 1998 A regulatory role for RIP140 in nuclear receptor activation. *Molecular Endocrinology* **12** 864–881. (doi:10.1210/mend.12.6.0123)
- Tseng CH 2012 Pioglitazone and bladder cancer in human studies: is it diabetes itself, diabetes drugs, flawed analyses or different ethnicities? *Journal of the Formosan Medical Association* **111** 123–131. (doi:10.1016/j.jfma.2011.10.003)
- Yang XJ & Gregoire S 2006 A recurrent phospho-sumoyl switch in transcriptional repression and beyond. *Molecular Cell* **23** 779–786. (doi:10.1016/j.molcel.2006.08.009)
- Ye J 2011 Challenges in drug discovery for thiazolidinedione substitute. *Yao Xue Xue Bao* **1** 137–142. (doi:10.1016/j.apsb.2011.06.011)
- Zamir I, Zhang J & Lazar MA 1997 Stoichiometric and steric principles governing repression by nuclear hormone receptors. *Genes and Development* **11** 835–846. (doi:10.1101/gad.11.7.835)

Received in final form 16 June 2014

Accepted 26 June 2014

Accepted Preprint published online 30 June 2014